Church, T. W., Weatherall, K., Correa, S., Prole, D., Brown, J. T., & Marrion, N. (2015). Preferential assembly of heteromeric small conductance calcium-activated potassium channels. *European Journal of Neuroscience*, *41*(3), 305-315. https://doi.org/10.1111/ejn.12789

Molecular & Synaptic Mechanisms

Preferential assembly of heteromeric small conductance calcium-activated potassium channels

Timothy W. Church, Kate L. Weatherall[1], Sonia A.L. Corrêa[2], David L. Prole[3], Jon T. Brown[4] and Neil V. Marrion

School of Physiology and Pharmacology, University of Bristol, Bristol BS8 1TD, United Kingdom

[1]Present address: MediTech Media Ltd, London, EC1V 9RU, UK.

[2]Present address: School of Life Sciences, University of Warwick, Coventry, CV4 7AL., UK.

[3]Present address: Department of Pharmacology, University of Cambridge, Cambridge, CB2 1PD, UK.

[4]Present address: University of Exeter Medical School, Hatherly Building, Exeter, EX4 4QJ, UK.

Running Title: Preferential assembly of heteromeric SK channels

Corresponding author:
Professor Neil V. Marrion
School of Physiology and Pharmacology, University of Bristol, Bristol BS8 1TD, United Kingdom; E-mail: N.V.Marrion@bris.ac.uk
Tel: +441173311401
Fax: +441173312288

Number of text pages: 20
Number of tables: 0
Number of figures: 7

Number of words:
Whole manuscript: 8065
Abstract: 232
Introduction: 499

Keywords: SK/ apamin/ species-specific/ channel/ assembly

Abstract

Activation of calcium-dependent SK channels regulates membrane excitability by causing membrane hyperpolarization. Three subtypes (SK1-3) have been cloned, with each subtype expressed within the nervous system. The location of channel subunits overlap, with SK1 and 2 subunits often expressed in the same brain region. We show that expressed homomeric rat SK1 subunits do not form functional channels, because subunits accumulate in the Golgi. This questions whether heteromeric channels could form with SK1 subunits. Co-expression of SK1 and SK2 subunits in HEK293 cells preferentially co-assembled to produce heteromeric channels with a fixed stoichiometry of alternating subunits. Expression in hippocampal CA1 neurons of mutant rat SK1 subunits (rat SK1(LV213/4YA)) that produce apamin-sensitive current changed the amplitude and pharmacology of the medium afterhyperpolarization (mAHP). Overexpression of rat SK1(LV213/4YA) subunits reduced the sensitivity of the mAHP to apamin, substantiating the preferential co-assembly of SK1 and SK2 subunits to form heteromeric channels. Species-specific channel assembly occurs as co-expression of human SK1 with rat SK2 does not form functional heteromeric channels. Replacement of two amino acids within the C-terminus of rat SK2 with those from human SK2 permitted assembly of heteromeric channels when co-expressed with human SK1. These data show that species-specific co-assembly is mediated by interaction between C-termini of SK channel subunits. The finding that SK channels preferentially co-assemble to form heteromeric channels suggests that native heteromeric channels will predominate in cells expressing multiple SK channel subunits.

Introduction

Molecular cloning of small-conductance calcium-activated potassium (SK, $K_{Ca}2$) channels from rat brain revealed three subtypes (SK1-3) (Kohler *et al*., 1996). Despite the lack of a therapeutic SK channel blocker, these channels have been suggested as a potential target for the treatment of dementia, depression and cardiac arrhythmias. The three subtypes display a partially overlapping regional and cellular expression pattern (Sailer *et al*., 2002; Sailer *et al*., 2004), with SK1 and SK2 showing a higher degree of co-localization than SK3 (Sailer *et al*., 2002; Sailer *et al*., 2004; Stocker & Pedarzani, 2000). Significant levels of SK1 and SK2 protein are present in numerous areas of the neocortex and the hippocampal formation (Sailer *et al*., 2004), with channel subunits found largely in postsynaptic dendritic and somatic regions (Ngo-Anh *et al*., 2005; Sailer *et al*., 2004).

SK channel subtypes are characterized by their sensitivity to the bee venom toxin apamin. SK2 is the most sensitive, followed by SK3 and SK1, but with some species variation as rat SK1 is insensitive (Kohler *et al*., 1996; Weatherall *et al*., 2010). Co-expression of different SK channel subunits in a heterologous expression system produces heteromeric channels that can be identified by their different sensitivities to block by apamin (Benton *et al*., 2003; Monaghan *et al*., 2004). Significant progress has been made in determining how SK current is inhibited by extracellular apamin and the implications of this for discriminating homomeric from heteromeric channel current (Lamy *et al*., 2010; Weatherall *et al*., 2011). For example, co-expression of a C-terminal chimeric rat SK1 channel subunit with wildtype rat SK2 subunits produced a current that was inhibited by apamin in a manner that indicated a single population of heteromeric channels (Weatherall *et al*., 2011). This suggests that SK channels prefer to form heteromeric rather than homomeric channels.

Wildtype rat SK1 subunits do not form functional channels when expressed in mammalian cell lines (Bowden *et al*., 2001; Benton *et al*., 2003; D'Hoedt *et al* 2004), with functional current only being obtained with a chimeric rat SK1 construct (D'Hoedt *et al* 2004; Weatherall *et al*., 2011). We show that the lack of functional wildtype rSK1 channel current results from rat SK1 subunits being accumulated in the Golgi apparatus and not being transported to the plasma membrane. Co-expression of rat and human isoforms of SK1 and 2 shows that both rat SK1-rat SK2 and human SK1-human SK2 preferentially express as heteromeric channels of a fixed stoichiometry, with no detectable levels of functional homomeric SK1 or SK2 channels. In contrast to previous findings, we find that the SK channel component contributing to the medium afterhyperpolarization in rat hippocampal CA1 neurons is mediated by homomeric SK2 channels. However, these channels prefer to co-assemble with another SK channel, as virally-mediated expression of a rat SK1 mutant produced only heteromeric SK channels. We also found differences in the heteromeric assembly between species, with human SK1 and rat SK2 not assembling as heteromeric channels. This species-specific assembly arises from a two amino acid difference within a XQMEX motif found in the C-termini of rat and human SK2 channel subunits.

Materials and methods

Constructs and cell culture

Wildtype rat SK2 (GenBank™ accession number NM_019314), human SK2 (GenBank™ accession number NM_021614.2) and human SK1 (GenBank™ accession number NM_002248.3) channel DNAs were subcloned into the mammalian plasmid expression vector pcDNA3 (Invitrogen, Paisley, UK). Wild-type rat SK1 and human SK3 were subcloned into the mammalian plasmid expression vector pFLAGCMV2 (Sigma-Aldrich, Poole, UK). Point

mutations in rSK2 [rSK2(TNAS)] were introduced using the QuikChange XL site-directed mutagenesis kit (Stratagene-Agilent, Stockport, UK) and subsequently confirmed by dye termination DNA sequencing. Human SK2 was a generous gift from Dr. Palle Christophersen (NeuroSearch A/K, DK). The ER marker pECFP-ER (Stephens *et al*., 2000) and pEGFP-C2 DNAs were purchased from Clontech (CA, USA). The marker for Golgi (pCMV-NAGFP (Shima *et al*., 1997)) was kindly provided by Dr. D. Stephens (University of Bristol).

Channels were transiently expressed in HEK293 cells, with cells maintained as described previously (Goodchild *et al*., 2009). Transient transfections of HEK293 cells were made using polyethyleneimine (Alfa Aesar, Inc.) by combining channel plasmid DNA with enhanced green fluorescent protein (EGFP) DNA in a ratio of 1:5 to 1:10 (maximal plasmid content: 1 μg). The ratio of co-expressed SK subunit plasmid DNAs was 1:1. Cells were used 24-48 hr after transfection.

Preparation of modified Sindbis virus encoding mutant rat SK1 subunits

Rat SK1(LV213/4YA) was first excised from the mammalian expression vector pFLAG-CMV2 (Weatherall *et al*, 2011) and subcloned into a pIRES2-EGFP vector. Rat SK1(LV213/4YA)-IRES2-EGFP was subsequently excised and sub-cloned into the linearized pSinRep5 (nsp2s) viral expression vector. Successful constructs were verified by DNA sequencing (Source BioScience, Oxford). Attenuated Sindbis virus (SINrep(nsP2S726)) was prepared and used as previously reported (Kim *et al*., 2004; Martin *et al*., 2007). Linearized pSinRep5-rat SK1(LV213/4YA)-IRES2-EGFP, pSinRep5-IRES2-EGFP and helper DHBB DNA (which encodes the structural proteins of the Sindbis virus) were transcribed *in vitro* and the recombinant RNA introduced into BHK cells by electroporation (Bio-Rad). The recombinant RNA encoding rat SK1(LV213/4YA)-IRES2-EGFP or IRES2-EGFP alone was

packaged and enveloped by the structural proteins produced by the helper DHBB RNA. The produced replication-incompetent pseudovirions were released into the extracellular medium, and harvested after 48 hours to be stored at -80°C.

Organotypic hippocampal slice preparation & Sindbis virus infection

Organotypic slices were prepared from 18-20 day old male Wistar rats as described previously (Stoppini *et al*., 1991). Rats were killed by cervical dislocation in accordance with Schedule 1 of the UK Home Office guidelines set out in the Animals (Scientific Procedures) Act 1986. All procedures were carried out in accordance with the UK Animal (Scientific Procedures) Act, 1986, and EU Directive 2010/63/EU. Furthermore all experimental procedures were reviewed by the University of Bristol Ethical Review Group (reference: UB/12/006). Horizontal 300 μM thick hippocampal slices were cut in ice-cold (~4˚C) sucrose-based saline solution (in mM): 189 sucrose, 10 glucose, 26 $NaHCO_3$, 3 KCl, 5 $MgSO_4.7H_2O$, 0.1 $CaCl_2$, saturated with 95 % O_2 and 5 % CO_2, pH 7.4, using a VT1000 S vibrating blade microtome (Leica Microsystems Ltd., Milton Keynes, UK). Slices were subsequently transferred to a storage chamber filled with artificial cerebrospinal fluid (aCSF) containing (in mM): 124 NaCl, 3 KCl, 24 $NaHCO_3$, 1.25 NaH_2PO_4.H2O, 1 $MgSO_4.7H_2O$, 10 D-glucose saturated with 95 % O_2 and 5 % CO_2, pH 7.4. Slices were washed twice with culture media containing MEM (Gibco) supplemented with (in mM): 50 $NaHCO_3$, 75 HEPES, 437 glutamine, 0.625 $CaCl_2$, 1.25 $MgSO_4.7H_2O$, 40 glucose, 0.425 ascorbic acid, with 12.5 % horse serum, 1 mg/ml insulin, and 100 units/ml penicillin with 100 μg/ml streptomycin (pH 7.28 with NaOH) (320 mOsm). Slices were washed a further two times in culture media without added penicillin/streptomycin. Slices were cultured at 37˚C (5 % CO_2) for 3 days, at the interface of the culture media without added antibiotics. Organotypic slices were infected with the Sindbis virus on the 2nd day in

culture by the droplet method, where the virus stock was diluted and a total of 1 µl virus solution was dropped over the slice. Slices were incubated with the virus for 24 hours.

Electrophysiology

Cell lines

Comparison of current amplitudes was accomplished using whole-cell recording from eGFP-positive cells bathed in a solution of composition: KAspartate (120 mM), KCl (20 mM), $MgCl_2$ (1.44 mM total, 1 mM free), HEPES (10 mM), EGTA (10 mM), $CaCl_2$ (6.19 mM total, 60 nM free) (pH 7.4). Fire-polished electrodes (3-5 MΩ) pulled from borosilicate glass contained: $KMeSO_4$ (120 mM), KCl (20 mM), $MgCl_2$ (1 mM free); HEPES (10 mM), EGTA (10 mM), Na_2ATP (3 mM) (pH 7.4). For a calculated free $[Ca^{2+}]$ of 60 nM, 6.19 mM $CaCl_2$ and 1.44 mM $MgCl_2$ was added, while for a calculated free $[Ca^{2+}]$ of 1 µM, 9.65 mM $CaCl_2$ and 2.34 mM $MgCl_2$ was used. HEK293 cells were voltage-clamped at 0 mV using an Axopatch 200A amplifier (Molecular Devices, CA, USA). Capacitance and series resistance compensation (>90%) was used throughout, with currents filtered at 1 kHz (8-pole low pass Bessel filter, Frequency Devices, CT, USA) and sampled at 5-10 kHz using Pulse (HEKA). SK-mediated currents were revealed by incremental 10 mV voltage steps of 1s duration. Currents were measured at the end of the 1 s pulse.

Outside-out macropatches excised from EGFP-positive cells were bathed in a solution of composition (in mM): 120 KCl, 10 HEPES, 10 EGTA, 6.19 $CaCl_2$ (calculated free $[Ca^{2+}]_i$, 60 nM), and 1.44 $MgCl_2$ (calculated free $[Mg^{2+}]_i$, 1 mM), pH 7.4, with ~40 mM KOH. SK current was activated by an internal $[Ca^{2+}]$ of 1 µM by filling electrodes, fabricated from KG-33 glass (Friedrich & Dimmock, Inc.), with a solution composed of (in mM): 120 KCl, 10 HEPES, 10 EGTA, 1.5 Na_2ATP, 9.65 $CaCl_2$ (calculated free $[Ca^{2+}]_i$ 1 µM), 2.34 $MgCl_2$ (calculated free

$[Mg^{2+}]_i$ 1 mM), pH 7.4 with ~40 mM KOH. For concentration-inhibition curves, solutions were exchanged using an RSC200 rapid switcher (Biologic). Expressed SK currents were recorded with an Axopatch 200A amplifier and visualized by a 1 s voltage ramp from −100 to +100 mV.

Organotypic hippocampal slices

Slices were continuously perfused (2-3 ml/min) at 33ºC, with aCSF supplemented with NBQX (10 μM) to inhibit spontaneous mini excitatory post-synaptic currents (EPSCs). Whole cell current-clamp recordings were made using fire-polished pipettes manufactured from borosilicate glass (1.5 mm O.D., 0.86 mm I.D.) containing (in mM): 125 $KMeSO_4$, 10 KCl, 10 NaCl, 20 HEPES, 2 MgATP, 0.3 NaGTP, 0.2 EGTA, set to pH 7.3, 280-285 mOsm (pipette resistance 3-5 MΩ). A liquid junction potential was experimentally measured (+13mV) and was compensated for during recording. Hippocampal CA1 pyramidal cells were visualised using an IR-LED mounted on an Axioskop2 microscope (Carl Zeiss). Membrane voltage was recorded in the bridge-balance mode of the MultiClamp 700A amplifier (Molecular Devices, Union City, CA, USA). Membrane voltage was filtered at 1.2 kHz (8-pole low pass Bessel filter) and sampled at 5 kHz using Pulse (HEKA Electronics, Lambrecht, Germany). The mAHP was elicited by 15 action potentials evoked by 15 2 ms current (2 nA) injections delivered at 50 Hz. Any cell that did not fire 15 action potentials was discarded. Using $MeSO_4$ as the non-conductive anion in the electrode solution is favourable for recording the mAHP, but appears to cause a progressive increase in neuronal input resistance (Cook Kaczorowski *et al* 2007). We found that the input resistance changed during the experiment, increasing by 9.7 ± 0.04 % (n=15) over 5 minutes. This increase in input resistance was observed together with a 4.5 ± 0.06 % (n=15) increase in the amplitude of the mAHP. This small increase occurred over the time required for apamin to inhibit the mAHP and was not considered to affect the

magnitude of inhibition by the toxin. All data was obtained from a membrane potential of -75 mV (maintained by current injection).

Immunofluorescence and microscopy

Transfected HEK293 cells were washed three times with warm HEPES-buffered saline (HBS) of composition: NaCl (119 mM), KCl (5 mM), $CaCl_2$ (2 mM), $MgCl_2$ (2 mM), HEPES (25 mM), glucose (30 mM) (pH 7.2), fixed and permeabilized with methanol at -20°C for 1 min and blocked for 15 min in 5% BSA diluted in HBS. Coverslips were then incubated for 90 min at room temperature with mouse anti-FLAG M_2 (1/100, Sigma; for the FLAG-tagged constructs) and rabbit anti-EGFP (1/100, Sigma) in 5% BSA (Sigma). Primary antibodies were visualized with labelled secondary antibodies (Molecular Probes: Alexa green 488 goat anti-rabbit, Alexa red 568 goat anti-mouse, 10 μg/ml^{-1}) in 5% BSA. Non-specific labelling was determined by secondary antibody alone. Immunofluorescent staining was imaged using a 63x oil immersion lens on a Zeiss LSM510 confocal microscope (Oberkochem, Germany). Fluorophores were excited with 488 or 568 nm wavelengths and emission from a single confocal plane was detected through 505-530 band-pass and 560 long-pass filters. Images were processed using Adobe Photoshop 6.0 (Adobe) and CorelDraw 12.0.

Data Analysis

For concentration-inhibition relationships, data points representing current block were fit with a variable slope Hill equation in the form:

$$\frac{I}{I_{cont}} = A_{min} + \frac{A_{max} - A_{min}}{1 + 10^{(Log_{IC50} - [X])n_h}}$$

where I_{cont} is the amplitude of current at −60 mV in the absence of drug, I is the amplitude of current observed at a given concentration of blocker [(X), expressed in logarithmic units], A_{min} is I_{min}/I_{cont}, A_{max} is I_{max}/I_{cont}, IC_{50} is the concentration of blocker that blocks 50% of the sensitive

current, and n_h is the Hill coefficient. The Wilcoxon signed rank test was used to determine if the Hill coefficients were significantly greater than unity.

Where data were best fitted by the sum of two Hill equations, the following equation was used:

$$I / I_{cont} = A_{min} + \left(\frac{A_{frac} - A_{min}}{1 + 10^{(LogIC_{50,a} - X) \times n_{h,a}}}\right) + \left(\frac{A_{max} - A_{min}}{1 + 10^{(LogIC_{50,b} - X) \times n_{h,b}}}\right)$$

A_{frac} is the amplitude of the current at the maximum of the high-sensitivity component I_{frac}/I_{cont}, $IC_{50,a}$ is the IC_{50} of the high-sensitivity component, $n_{h,a}$ is the Hill coefficient of the high-sensitivity component, $IC_{50,b}$ is the IC_{50} of the low-sensitivity component, and $n_{h,b}$ is the Hill coefficient of the low-sensitivity component. Data were fit with the equation with the R^2 value closest to unity. Analysis of the mAHP was carried out using custom written MatLab scripts (The MathWorks company).

All numerical values are expressed as mean ± S.E.M. This study compares the sensitivity and degree of positive co-operativity of inhibition of SK channels by apamin. Statistical comparison of IC_{50} and Hill coefficient values from expression studies was carried out using the unpaired, two-tailed Student's *t* test with a directional hypothesis. In contrast, statistical comparison of inhibition of mAHP amplitude by apamin was performed using the paired two-tailed Student's *t* test with a directional hypothesis. Statistical analysis was performed using Prism 5.02 (GraphPad Software) and representative traces were drawn using Origin 6.1 (Microcal Software).

All salts were purchased from Sigma-Aldrich except HEPES, which was obtained from Merck Serono (Feltham, UK). Apamin and NBQX were purchased from Tocris Biosciences (Bristol,

UK). Apamin and NBQX solutions were prepared on the day of experiments from a frozen stock of 100 μM in water for apamin and a frozen stock of 10 mM in DMSO for NBQX.

Results

Subcellular location of expressed rat SK1 subunits in HEK293 cells

Expression of homomeric rat SK1 subunits fails to produce functional current (Bowden *et al*., 2001; Benton *et al*., 2003; D'Hoedt *et al* 2004). Co-expression of rat SK1 subunits and markers for the endoplasmic reticulum (ER) and Golgi in HEK-293 cells shows that rat SK1 protein resides within the ER, with very little migrating to the Golgi within 8 hours of expression (Figure 1A). A similar subcellular expression pattern is observed for human SK3 subunits, except that pronounced labelling is observed at the cells' edge (Figure 1C). Expressed rat SK1 subunits are observed throughout the ER, and accumulate within the Golgi 22 hours after transfection (Figure 1B). This subcellular localization is different from that observed for the human SK3 subunit, which shows protein expression within both the ER and Golgi and clear labelling at the cells' edge (Figure 1C & D). These data are consistent with the lack of functional rat SK1-mediated current and the large Ca^{2+}-activated human SK3-mediated current observed in whole-cell recording from transfected HEK293 cells (Figure 1E).

Reduced incubation temperatures have also been demonstrated to rescue expression of trafficking-defective HERG, CFTR and IK channels (Denning *et al*., 1992; Jones *et al*., 2004; Zhou *et al*., 1999). Maintaining cells transfected with rat SK1 at a reduced temperature of 27°C for 24 hrs (following a 24 hr transfection period) prior to recording fails to rescue functional current (Figure 1E). Exposure of transfected cells to the ER Ca^{2+}-ATPase inhibitor thapsigargin can rescue expression of trafficking defective HERG channels (Delisle *et al*.,

2003). Incubation of rat SK1-transfected HEK293 cells with thapsigargin (1μM) for 4-6 hrs prior to recording has no effect on expressed current level (Figure 1E). Other treatments attempted to rescue rat SK1 expression and provide functional channels, such as incubation of cells with chloroquine (Zhou *et al*., 1999), tacrine (Delisle *et al*., 2003) or dequalinium (Stocker & Pedarzani, 2000) also have no effect on expressed current level (Figure 1E).

Co-expression of rSK1 and rSK2 in HEK293 cells produces heteromeric channels

The retention of rSK1 subunits in the Golgi suggests that HEK293 cells lack a chaperone to export protein from the Golgi to the plasma membrane. Functional rat SK1 current is observed in transfected hippocampal neurons, suggesting that these neurons possess the necessary machinery to process this subunit (Correa *et al*., 2009). However, the overlap of expression of SK1 and 2 subunits suggests that it might be more common for cells to express heteromeric channels. Co-expression of these subunits in HEK293 cells produces current, but the stoichiometry of the functional channels was unknown (Benton *et al*., 2003). In control experiments rSK2 expressed alone produces inward rectifying currents that are sensitive to apamin in agreement with previous reports (IC_{50} 71.4 ± 14.6 pM, n = 6, Figures 2A and C) (Lamy *et al*., 2010; Nolting *et al*., 2007; Weatherall *et al*., 2010; Weatherall *et al*., 2011). Co-expression of rat SK1 and rat SK2 subunits produces inward rectifying currents that are also sensitive to apamin, with the concentration-inhibition curve being well fit with a single Hill equation. In contrast to the inhibition of homomeric rat SK2 currents, heteromeric rat SK1-SK2 current was approximately 6-fold less sensitive to block by apamin (IC_{50} 445 ± 65 pM, n = 6, Figures 2B and C). The concentration-inhibition relationship of homomeric rat SK2-mediated current displays positive co-operativity, by exhibiting a fitted Hill slope that is significantly greater than unity (1.98 ± 0.28, n = 6, $P < 0.02$) (Lamy *et al*., 2010; Weatherall *et al*., 2011). This co-operativity arises by interaction between SK2 subunits within the channel

tetramer (Lamy *et al*., 2010). Inhibition of heteromeric rat SK1-SK2 current exhibited a Hill slope close to unity (1.02 ± 0.12, n = 6, $P > 0.05$). These data indicate that heteromeric rat SK1-SK2 current arises from co-assembled channels of fixed stoichiometry, with the rat SK2 subunits being non-adjacent within the tetramer.

Human SK1 and rat SK2 do not assemble as heteromeric channels

Unlike homomeric rat SK1 channels, human SK1 subunits form functional homomeric channels when expressed in mammalian expression systems (Figure 3A)(Dale *et al*., 2002; Shah & Haylett, 2000; Strobaek *et al*., 2000; Weatherall *et al*., 2011). Inward rectifying currents from cells co-expressing human SK1 and rat SK2 were reduced by apamin, with the resultant apamin concentration-inhibition curve being best fit by the sum of two Hill equations (Figures 3B and C). The high sensitivity component displayed an IC_{50} of 104 ± 25 pM and n_h of 2.67 ± 0.48 (n = 6), while the low sensitivity component displayed an IC_{50} of 1.82 ± 0.25 nM and n_h of 1.61 ± 0.32 (n = 6). The IC_{50} of the high sensitivity component is not significantly different from inhibition of expressed rat SK2 homomeric channel-mediated current (IC_{50} 78.5 ± 16.3 pM, $P > 0.40$, Figure 2D). The IC_{50} of the low sensitivity component is not significantly different from that observed for homomeric human SK1-mediated current (IC_{50} 1.51 ± 0.25 nM, n = 6, $P > 0.40$, Figures 3A and D). These data indicate that the biphasic curve describes inhibition of homomeric human SK1 and homomeric rat SK2 channels and that heteromeric channels were not formed.

Heteromeric channel assembly is species-specific

The lack of heteromeric channels being formed between human and rat SK channel subtypes either suggests that human isoforms of SK channels do not form heteromeric channels or co-assembly is species-specific. Homomeric human SK2 channel current has a sensitivity to

apamin that is similar to homomeric rat SK2-mediated current (IC_{50} 107 ± 11 pM, n = 6, Figures 4A and C), while homomeric human SK1-mediated current is inhibited by apamin with an IC_{50} of 1.51 ± 0.25 nM (n = 6). Co-expression of human SK1 and SK2 subunits produces inward rectifying current that is inhibited by apamin with a sensitivity that is intermediate to both homomeric human SK1 and human SK2 channel current (human SK1-SK2, IC_{50} 295 ± 35 pM, n = 6, Figures 4B and C). This concentration-inhibition relationship is best fit by a single component Hill coefficient, suggesting a single population of channels. The Hill slope for inhibition of heteromeric human SK1-SK2 current is not significantly greater than unity (n_h 1.11 ± 0.12, $P > 0.30$), which is similar to that seen for homomeric human SK1 current (n_h 0.98 ± 0.04, $P > 0.40$) but is in contrast to the Hill slope seen for homomeric human SK2-mediated current (n_h 1.24 ± 0.06, $p < 0.02$). The positive co-operativity exhibited with inhibition of homomeric rat and human SK2 channel current by apamin arises from interaction between subunits (Lamy *et al*., 2010). Although positive co-operativity is seen with both rat and human homomeric SK2 channels, inhibition by apamin displays different degrees of co-operativity even though the sensitivity to inhibition (IC_{50}) is comparable. This illustrates that as expected, there is no correlation between sensitivity and degree of co-operativity of inhibition.

These data show that human SK channel subunits can co-assemble to form functional heteromeric channels. Importantly, the data shows that they prefer to form heteromers, as the concentration-inhibition relationship indicates a single population of heteromeric channels with no evidence of homomeric channels. The lack of co-operativity of inhibition by apamin indicates that these heteromeric channels assemble with non-adjacent human SK2 subunits.

Residues within the C-terminus determine species-specific formation of heteromers

Sequence alignment of the C-termini of rat and human SK2 shows two residues that differ between the two isoforms of the channel, Thr^{543}/$Asn^{547}$and Ala^{542}/Ser^{546} in rat SK2 and human SK2 respectively (Figure 5). These residues are outside the calmodulin binding domain (CaMBD) and predicted coiled-coil domains within the C-terminus that are implicated in the tetramerization of SK channels (Tuteja *et al*., 2010). Mutation of both residues in rat SK2 to the corresponding residues in human SK2 [rat SK2(TNAS)] yielded a subunit with an identical C-terminus to human SK2. Co-expression of human SK1 with rat SK2(TNAS) produces inward rectifying currents, whose sensitivity to inhibition by apamin is intermediate to homomeric human SK1 and rat SK2, with an IC_{50} that is not significantly different from inhibition of the heteromeric human SK1-SK2 channel current (329 ± 89 pM, n = 6, $P > 0.70$, Figures 6A & B). Preferential formation of heteromeric channels occurs when human SK1 and rat SK2(TNAS) are co-expressed, as the concentration-inhibition curve is best fit with a single component relationship. Finally, inhibition by apamin does not exhibit positive co-operativity, because the Hill coefficient is close to unity (n_h 0.85 ± 0.09, $P > 0.07$). These data show that like human SK1-SK2 heteromers, heteromeric human SK1-rat SK2(TNAS) channels assemble with non-adjacent rSK2(TNAS) subunits.

Formation of heteromeric channels in hippocampal neurons

The identity of the channel subtypes that underlie the medium afterhyperpolarization (mAHP) in hippocampal CA1 neurons is unclear, with the activation of SK-, M- and H-current being suggested (Stocker *et al*., 1999; Gu *et al*., 2005; Lima & Marrion, 2007). The firing of 15 action potentials evoked the mAHP, which was inhibited by 21.8 ± 6.3 % (n = 15; $P < 0.009$) by application of apamin (100 nM) (Figure 7A). This SK channel-mediated component of the mAHP is inhibited by apamin with an IC_{50} of 52 pM, which is indicative of the current being carried by homomeric SK2 channels (Figure 7C). Overexpression of the apamin-sensitive

mutant rat SK1(LV213/4YA) subunit (Weatherall *et al*., 2011) in CA1 neurons produces a larger mAHP (rat SK1(LV213/4YA) -6.5 ± 0.83 (n = 9) vs eGFP control -3.84 ± 0.29 (n = 15) mV), with more of the mAHP being sensitive to apamin (% block by 100 nM apamin: rat SK1(LV213/4YA) 32.6 ± 2.5 (n = 9) vs eGFP control 21.8 ± 6.3 (P = 0.0002, n = 15)) (Figure 7B). The mutant rat SK1(LV213/4YA) subunit displays identical pharmacology to human SK1, being inhibited by apamin with an IC_{50} of 1.4 nM (Weatherall *et al*., 2011). This is because rat and human SK1 subunits have identical outer pore sequences and the amino acid substitutions (LV213/4YA) in the S3-S4 extracellular loop turn rat SK1 into an apamin-sensitive channel (Weatherall *et al*., 2011). In addition, human and rat SK2 display the same sensitivity to apamin (Weatherall *et al*., 2011; Figure 4A). Co-expression of human SK1 and SK2 produces a current that is blocked by apamin with an IC_{50} of 295 pM (Figures 4B and C). The sensitivity of hSK1-SK2-mediated current mirrors that we observe in hippocampal neurons expressing rat SK1(LV213/4YA), with the SK channel-mediated component of the mAHP being inhibited by the toxin with an IC_{50} of 220 pM (n = 9) (Figure 7C). Importantly, a single component inhibition relationship is observed, indicating that there is a single population of heteromeric apamin-sensitive channels after expression of rat SK1(LV213/4YA).

Discussion

The retention of rat SK1 subunits in the ER and Golgi apparatus of HEK293 cells suggests that a chaperone is required to transport this subunit to the plasma membrane. This chaperone might be absent in HEK293 cells, as expression of these subunits in hippocampal neurons produces functional current (Correa *et al*., 2009). However, rat SK1 forms heteromeric current with rat SK2 subunits in HEK293 cells (Figure 2). It seems possible that the extensive co-

localization of SK1 and SK2 channel subunits within the central nervous system is used to form heteromeric channels, and that the SK2 subunit is the chaperone.

Hippocampal CA1 pyramidal neurons express both SK1 and SK2 channel subunits, but with different subcellular locations (Bowden *et al.*, 2001; Sailer *et al.*, 2002; 2004). There is controversy regarding the contribution of channel subtypes to activation of the mAHP. It has been reported that apamin blocks a significant proportion of the mAHP in these neurons, indicating that activation of SK channels is a significant contributor to the mAHP (Stocker *et al.*, 1999; Pedarzani *et al.*, 2005; Cook Kaczorowski *et al.*, 2007). The block of the mAHP by apamin displayed an intermediate sensitivity (IC_{50} ~ 480 pM, Stocker *et al.* 1999). This suggests that if SK channel activation underlies the afterpotential the channel is likely to be a heteromer of SK1 and SK2, as the sensitivity to apamin is mirrored by that seen from inhibition of expressed rSK1-rSK2 current (Figure 2C). In contrast, it has been reported that SK channel activation only occurs under voltage clamp and the afterpotential results from activation of M-current and deactivation of H-current (Gu *et al*., 2005). We found that only approximately 25% of the mAHP is contributed to by activation of SK channels, and that the SK channel concerned is likely to be homomeric SK2. Our unpublished data indicates that the remainder of the mAHP recorded at -75 mV (as in this study) results from H-current. The observed involvement of SK channel activation in generation of the mAHP is consistent with others (Stocker *et al.*, 1999; Pedarzani *et al.*, 2005; Cook Kaczorowski *et al.*, 2007), but not Gu *et al* (2005). However, the finding that activation of homomeric SK2 channels underlies the mAHP is not agreement with Stocker *et al* (1999). Any differences might arise from our use of organotypic cultured hippocampal slices, but it is clear that progress is required before the role of SK channel activation in the generation of the mAHP is resolved.

Over-expression of rat SK1(LV213/4YA) subunits augmented the amplitude of the mAHP. We used the mutant rat SK1 subunit to determine whether all expressed SK channel subunits were incorporated into heteromeric channels. This is possible because the mutant rat SK1 subunit produces functional homomeric channels that are sensitive to apamin, displaying a sensitivity to block by apamin that is identical to homomeric human SK1 channel current (Weatherall *et al.,* 2011). The wildtype rat SK1 subunit produces functional channels that are resistant to block by apamin (Weatherall et al., 2011), making resolution of homomeric channels difficult in the mixed background of conductances that underlie the mAHP in our organotypic hippocampal slice preparation (most being insensitive to apamin). The lack of homomeric rat SK1(LV213/4YA)-mediated current in virally-transformed CA1 neurons supports the proposal that SK1 could be a chaperone for SK2 subunits. Co-expression of the mutant rSK1 subunits within a heterologous expression system indicates that they prefer to co-assemble to form functional heteromeric channels. This is clearly also the case in neurons, as all apamin-sensitive current was derived from a single population of channels. Our data suggest that these preferred heteromeric channels possess a fixed stoichiometry and subunit arrangement, where subunits alternate in identity within the tetramer. A similarly fixed arrangement of channel subunits has been suggested for heteromeric $K_{ir}4.1$-$K_{ir}5.1$ channels (Pessia *et al*., 1996). A fixed stoichiometry and subunit arrangement has been reported for transient receptor potential channels, where co-expression of TRPP2 and TRPC1 subunits formed heteromeric channels with a 2:2 stoichiometry and subunits arranged alternately within the tetramer (Kobori *et al*., 2009; Stewart *et al*., 2010). In contrast, it has been proposed that heteromeric KCNQ (Kv7) channels display variable stoichiometry, with subunits arranged randomly (Steward *et al*., 2012). These data indicate that a fixed stoichiometry and arrangement of subunits within a channel tetramer might be common and would suggest that

diversity of potassium and other channels might not be as large as originally thought (Jan & Jan, 1990).

SK channels have been proposed to assemble via coiled-coil domains present in the C-terminus of each subunit (Tuteja *et al*., 2010). The finding that mutation of two amino acid residues within the C-terminus of rat SK2 enables formation of heteromeric channels with human SK1 subunits, supports the proposal that SK channel subunits assemble via interactions between C-termini. Coiled-coil domains are implicated in the assembly of a number of receptor and channel subtypes (; Jenke *et al*., 2003; Schwake *et al*., 2006; Mei *et al*., 2006) and consist of a heptad repeat sequence, with small hydrophobic residues in the first and fourth positions, and large polar residues in the fifth and seventh positions. There is a dominant predicted coiled-coil domain within the C-terminus of rat SK2 (residues 496-515), with a similar domain predicted in comparable positions within human SK2, human and rat SK1, and human SK3 (Tuteja *et al*., 2010). Mutation of two amino acid residues within the C-terminus of rat SK2, but outside the predicted coiled-coil domains, enables heteromeric channels to form between rat and human isoforms. These are the only two residues within the C-termini that differ between rat and human SK2 subunits. It is not known how these residues dictate the inability to form heteromeric channels across species. The residues within rat SK2 (threonine and asparagine) are bulkier than the corresponding alanine and serine found in human SK2. It is possible that the larger residues in rat SK2 prevent interaction of coiled-coil domains with their counterparts in human SK2.

The observation that SK channel assembly is species-specific indicates that caution is required when studying SK channel heteromerization. Some studies in the past have used rat and human SK channels with the assumption that heteromeric channels would form (eg: Ishii *et al*., 1997;

Roncarati *et al*., 2001; Monaghan *et al*., 2004). In some cases, the assembly of heteromeric SK channels was demonstrated by concentration-inhibition curves that display a significantly lower Hill coefficient than seen with homomeric channels (Monaghan *et al*., 2004). This is observed if the current is composed of two homomeric channel populations (as observed for apamin in this study), and does not represent the presence of different stoichiometries of heteromeric channels (Monaghan *et al*., 2004; Ishii *et al*., 1997).

The preferential co-assembly of heteromeric SK channels has important implications for their therapeutic targeting, and suggests that advantage is taken of the overlapping protein expression pattern of SK channel subunits in brain and heart. Tetrameric SK channels fold so that the extracellular loop between transmembrane segments S5 and S6 is donated to the adjacent subunit (Weatherall *et al*., 2011). Compounds are required to bind both the channel outer pore and the extracellular loop between transmembrane segments S3 and S4 to obtain subtype-selective inhibition (Lamy *et al*., 2010; Weatherall *et al*., 2011). High affinity inhibition that displays positive co-operativity can only be produced when the compound binds to both the S3-S4 extracellular loop and the outer pore of adjacent subunits (Weatherall *et al*., 2011). We show that heteromeric SK1-SK2 channels possess a fixed stoichiometry of 2:2, where subunits are arranged alternately. A compound would be required to bind to the extracellular loop of SK1 and the outer pore of SK2 or *vice versa* to inhibit a heteromeric SK1-SK2 channel. It is clear that this approach will enable the design of heteromeric subtype-specific inhibitors that will not affect homomeric channels and present few side effects.

Acknowledgements

The work was supported by both the Medical Research Council (UK) and a grant from the Belgian Science Policy Interuniversity Attraction Poles program. We wish to thank Prof. Vincent Seutin and Dr. Jane Hancock for critical reading of the manuscript. We are indebted to Prof. Jeremy Henley and Mr. Philip Rubin for invaluable assistance in the construction of the Sindbus virus constructs.

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Figure 1 Intracellular accumulation of rat SK1 subunits expressed in HEK293 cells. ***(A-D)*** Time course of the subcellular location of expressed pFLAG-tagged rat SK1 or human SK3 subunits. ***A***, Co-expression of either the ER marker pECFP-ER (green, above) or the Golgi marker pCMV-NAGFP in (green, below) and rat SK1 subunits (red) showed a predominantly ER location for the channel subunits within 8 hours after transfection. ***B***, Expressed rat SK1 subunits (red) migrated to the Golgi apparatus 22 hours after transfection, with the accumulation of channel subunits clearly observed. ***C***, Expression of human SK3 subunits (red) was throughout the cell 8 hours after transfection, with subunits distributed in both the ER and Golgi (green). ***D***, Human SK3 subunits (red) were distributed in both the ER and Golgi apparatus (green) 22 hours after transfection, with peripheral staining being apparent. Scale bar 5 μm. ***E***, Current density (pA/pF) recorded at –30 mV from cells expressing either rat SK1 or human SK3 subunits (relevant n given in brackets). Whole-cell recording with either 60 nM or 1 μM free Ca^{2+} in the electrode solution showed that no Ca^{2+}-dependent current was apparent 24 hours after transfection with rat SK1 subunits. Incubation of transfected cells at 27^{o}C to aid expression of rat SK1 subunits had no effect, nor did incubation of cells with the Ca^{2+}-ATPase inhibitor thapsigargin (1 μM), the blockers tacrine (300 μM) or dequalinium (10 μM), or the inhibitor of endocytosis chloroquine (100 μM). In contrast, a large Ca^{2+}-dependent current was observed in cells transfected with human SK3 subunits (note break in the abscissa).

Figure 2 Co-expression of rat SK1 and SK2 subunits produces heteromeric channels. ***A,B***, representative examples of outside-out macropatch currents derived from voltage ramps from -100 to 100 mV imposed on voltage clamped HEK293 cells expressing rat SK2 (***A***) and co-expressing rat SK1 and SK2 (***B***) subunits in control conditions and increasing concentrations of apamin. (***C)*** concentration-inhibition relationships for apamin inhibition of expressed rat SK2 and co-expressed rat SK1-SK2 current.

Figure 3 Co-expression of human SK1 and rat SK2 subunits does not produce heteromeric channel current. ***A,B***, representative examples of outside-out macropatch currents derived from voltage ramps from -100 to 100 mV imposed on voltage clamped HEK293 cells expressing human SK1 (***A***) and co-expressing human SK1 and rat SK2 (***B***) subunits in control conditions and increasing concentrations of apamin. ***C***, Concentration-inhibition relationships for apamin inhibition of expressed rat SK2, human SK1 and co-expressed human SK1-rat SK2 current. ***D***, Bar chart depicting apamin IC_{50} values for inhibition of rat SK2-, human SK1- and the high sensitivity and low sensitivity components of human SK1 co-expressed with rat SK2-mediated current. $IC_{50,a}$ was not significantly different from inhibition of homomeric rat SK2-mediated current ($p > 0.05$) and $IC_{50,b}$ was not significantly different from inhibition of homomeric human SK1 current ($p > 0.05$).

Figure 4 Co-expression of human SK1 and SK2 subunits produces heteromeric channel current. ***A,B***, representative examples of outside-out macropatch currents derived from voltage ramps from -100 to 100 mV imposed on voltage clamped HEK293 cells expressing human SK2 (***A***) and co-expressing human SK1 and human SK2 (***B***) subunits in control conditions and increasing concentrations of apamin. ***C***, Concentration-inhibition relationships for inhibition by apamin of expressed human SK1-, human SK2- and co-expressed human SK1-SK2-mediated current.

Figure 5 Alignment of the cytosolic C-terminal region of rat SK2 and human SK2. Predicted CaMBD regions are boxed in grey (Schumacher *et al*., 2001; Xia *et al*., 1998). Predicted coiled-coil domains are indicated in dashed boxes, with significant small hydrophobic residues

of the heptad repeats in dark grey (Tuteja *et al*., 2010). Residues differing between the two channel isoforms are boxed and in boldface type.

Figure 6 Point mutation of the residues Thr^{543} and Asn^{547} of rat SK2 to the corresponding Ala and Ser of human SK2 permits the formation of heteromeric channels with human SK1. ***A***, Representative example of outside-out macropatch currents derived from voltage ramps from -100 to 100 mV imposed on a voltage clamped HEK293 cell co-expressing human SK1 and rat SK2(TNAS) subunits in control conditions and increasing concentrations of apamin. ***B***, concentration-inhibition relationships for apamin inhibition of expressed human SK1, rat SK2 and co-expressed human SK1-rat SK2(TNAS) current.

Figure 7. Formation of heteromeric SK channels in hippocampal CA1 neurons. Representative membrane voltage traces of the mAHP evoked in control eGFP-expressing ***(Ai)*** and rat SK1(LV213/4YA)-expressing ***(Bi)*** neurons (elicited by 15 action potentials evoked by 2 ms current injections delivered at 50 Hz). Control – black, apamin (100 pM) – grey, apamin (100 nM) – light grey. ***Aii,Bii***, Evoked AHPs are displayed on a $\log_{10}$ time scale to illustrate how the mAHP is discriminated. The mAHP amplitude was taken from the peak voltage deflection recorded 0-100 ms post-burst. ***C***, Fractional block of the apamin-sensitive component of the mAHP. Overexpression of the rat SK1(LV213/4YA) subunit changed the sensitivity of the mAHP to apamin, with the SK channel-mediated component being inhibited with an IC_{50} of 52 pM in eGFP-expressing neurons and an IC_{50} of 220 pM in rat SK1(LV213/4YA)-expressing cells (numbers in parentheses correspond to n).